

Characterization of *bla_{VIM}* & *bla_{IMP}* in Imipenem non-Susceptible multidrug resistant *Pseudomonas aeruginosa* in a tertiary care hospital in eastern India.

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Abstract:

Background: Metallo beta lactamases in *Pseudomonas aeruginosa* are often detected phenotypically. In our institution we frequently encountered such incidence but no report about the genotypic character of such isolates was available.

Objective: Thus present study aimed at characterization of the MBL encoding gene in imipenem non-susceptible multi drug resistant *Pseudomonas aeruginosa*, isolated from clinical samples.

Materials & methods: 98 Non-duplicate consecutive imipenem non susceptible strains of *Pseudomonas aeruginosa* were selected from different clinical samples. Combined disc diffusion test with imipenem - EDTA (10µg+750µg) combined disc was used to screen for MBLs activity. PCR for identification of *bla_{VIM}* & *bla_{IMP}* gene was performed.

Result: Combined disc test phenotypically screened 51 out of 98 imipenem non-susceptible isolates of *Pseudomonas aeruginosa* with MBL activity and 47 as MBL negative. *bla_{VIM}* gene was detected in 9 out of 98 isolates by PCR; no other MBL encoding gene was detected. The existence of *bla_{VIM}* gene explained imipenem non-susceptibility in multidrug resistant *Pseudomonas aeruginosa* and its prevalence was 9.18% (9 out of 98) in our healthcare set up at the time of study.

Keywords: Metallo-β-lactamase (MBL), multidrug resistance, *Pseudomonas aeruginosa*, *bla_{VIM}* & *bla_{IMP}*

I. Introduction

MBLs are increasingly been recognized as most important resistance determinant in Gram negative bacilli from different parts of the world including areas of low carbapenem resistance [1,2]. These group of enzymes hydrolyse most of the beta lactam antibiotics including carbapenems which are the backbone of treatment for multidrug resistant Gram negative bacilli specially *Pseudomonas aeruginosa*, an organism well known for its intrinsic and acquired resistance to many antimicrobial agents. So MBL in *P.aeruginosa* is most worrisome condition to treat.

Genes encoding MBLs are of two types - chromosomally mediated and transposable genetic element mediated [3]. Transposable MBL gene IMP, VIM & GIM are found as gene cassettes in class I integron [4]. The dissemination of MBL gene is thought to be driven by the regional consumption of carbapenem or cephalosporins [5]. Acquired MBLs, encoded by integron borne mobile gene cassettes, are often accompanied by resistant determinants for other group of antibiotics. Thus multiple drug resistance is seen in MBLs producing isolates [6]. At present acquired MBLs have spread worldwide. Different types of genes e.g. IMP, VIM, SPM & GIM have been identified in *P.aeruginosa*. IMP and VIM have been reported from various parts of the world but SPM & GIM are restricted to Brazil and Germany respectively [7].

In our tertiary care hospital in eastern India we encountered imipenem non susceptible multi drug resistant *P. Aeruginosa* in clinical samples from various sources. So our study aimed at genotypic characterization of those isolates. The specific objective of this study on resistance determining genes was to know the prevalent gene conferring MBL activity.

II. Materials & Methods

2.1) Clinical specimens

From clinical samples like pus, blood, sputum, urine, broncho-alveolar lavage fluid, tracheal aspirate and urinary catheter tip sent to department of Microbiology, non-duplicate consecutive isolates of *Pseudomonas aeruginosa* were selected from January 2012 to September 2012. Isolates with resistance to imipenem were

selected for study and those susceptible to imipenem were excluded. These isolates were also resistant to 2 or more different classes of antibiotics from the list of ceftazidime, piperacillin, ciprofloxacin, amikacin or tobramycin. The study protocol complied with the Declaration of Helsinki and was approved by institution's ethics committee.

2.2) Identification of strains: Isolates were confirmed as *Pseudomonas aeruginosa* by standard laboratory tests from patients' sample. Gram staining, motility test, growth in selective medium of cefrimide agar with 0.5% nalidixic acid (Himedia M04-100G), pigment production, growth at 42^oc, oxidation-fermentation test, catalase and oxidase test, nitrate reduction test and dihydrolysis of arginine were the phenotypic tests performed.

2.3) Antibiogram: Antibiotic susceptibility test by Kirby-Bauer disk diffusion method with six classes of different antibiotics was performed according CLSI 2011 guidelines. These were imipenem (10 µg), ceftazidime (30µg), piperacillin (100µg), tobramycin (10µg), gentamicin(10µg) and ciprofloxacin(5µg). ATCC *Pseudomonas aeruginosa* 27853 was the control for antibiotic susceptibility test.

2.4) Phenotypic identification of MBL activity by combined disc test :

The combined disc test was performed following method described by J. Pitout[8,11] and Yong D et al [9,12]. Briefly, a bacterial suspension of test strain in peptone water was incubated at 37^o C for 2 hrs; turbidity was matched with 0.5 McFarland turbidity standard and then inoculated in Muller-Hinton agar as lawn culture. Imipenem (10µg) disc alone and imipenem –EDTA(10µg+750µg) combined disc were placed at 15 mm distance from centre to centre to detect MBL production by ≥ 7mm increase in zone of inhibition with combined disc as compared to imipenem alone. MBL producing laboratory isolate of *Klebsiella* spp. was used as positive control and *Pseudomonas aeruginosa* ATCC 27853 as negative control for combined disc test.

2.5) Detection of *bla_{vim}* and *bla_{IMP}* gene in *Pseudomonas aeruginosa*:

Genomic DNA purification spin kit(HipurATM MB 505) provided a fast and easy method for purification of total DNA. Duplex Polymerase chain reaction was used for identification of *bla_{vim}* and *bla_{IMP}* gene. Briefly, 100 µg bacterial DNA was amplified with DNA polymerase (AmpliTaq Gold; Applied Biosystems, Inc. [ABI], Foster City, CA). In PCR reaction master mixtures, contained 1X PCR buffer (Applied Biosystems) 5µl, 2 mM MgCl₂, 0.25 mM each dNTP, 20 picomoles of each forward and reverse primer, Taq polymerase 1 µl and water 26 µl to prepare 45 µl of master mix for one sample to which 5 µl DNA template was included. In Table 1 detail of the primers used is given [7,8,12]

The PCR reactions were performed in the following conditions: 37°C for 10 minutes, 95°C for 4 minutes, followed by 30 cycles of 94°C for 1 minute, annealing temperature (Table-1) for 1 minute, 72°C for 1.5 minutes, and then 72°C for 10 minutes for the final extension. The amplified products were separated by electrophoresis on 2% agarose gel stained with 0.5 mg/mL ethidium bromide and visualized and photographed under a UV transilluminator. *Pseudomonas aeruginosa* ATCC 27853 was considered as negative control for the genotypic study. The first isolate (P32) showing amplicons of molecular weight between 300bp & 400bp (Figure 1) corresponding *bla_{vim}* gene amplicon, was considered as positive control for PCR. All strains were further studied to confirm absence of *bla_{SPM1}* and *bla_{GIM}* gene using the following condition: 95°C for 5 minutes, followed by 30 cycles of 95°C for 1 minute, Ta^o for the specific primers (Table 1) for 1 minute, 68°C for 1 minute, and then 68°C for 5 minutes for the final extension. Phenotypically MBL positive isolates were the test strains and MBL negative isolates were control strains used for Duplex PCR.

2.6) Statistical Analysis

Comparative study of two qualitative tests– disc potentiation test (screening test) and PCR for gene detection(gold standard test) was done by Chi-square test. The results of the test was analysed using “Graph pad” statistical software.

III. Results

Total 98 non-duplicate consecutive strains of *Pseudomonas aeruginosa*, resistant to imipenem and 2 or more antibiotics from different classes were selected for study during the period of nine months from January 2012 to September 2012. The antibiotic susceptibility test by Kirby –Bauer disc diffusion method as suggested by CLSI guidelines 2011 for *P.aeruginosa* was performed. All isolates appeared resistant to imipenem and other two, three, four or five different classes of antibiotics (Table 2).

3.1) Phenotypic screening of MBL activity by Combined disc Test:

Imipenem non-susceptible 51 strains isolated mainly from pus sample showed ≥ 7 mm increase in zone of inhibition with imipenem-EDTA combined disc as compared to imipenem alone and were considered

as MBL positive. The 47 strains which showed < 7 mm comparative increase or no increase in zone of inhibition or the total zone of inhibition <13mm with imipenem- EDTA disc were considered as MBL negative.

3.2) Result of Duplex PCR:

With extracted DNA of 98 isolates duplex PCR was performed for *bla_{vim}* and *bla_{IMP}* gene. Out of 51 'study strains' eight (P32, P40, P61, P65, P76, P84, P89, P90, P96) isolates showed the amplicon size between 300bp & 400bp in DNA ladder (Figure 1). This finding corresponds to *bla_{vim}* gene of 382 bp. Among forty seven control strains one sample (P61) was positive for *bla_{vim}* gene. As no amplicon of 587bp was detected so it was concluded that *bla_{IMP}* gene was absent in all the isolates (Table 3).

All isolates harbouring *bla_{vim}* gene were identified in pus samples collected from patients of different departments like surgery, orthopaedic, burn, ENT etc. (Table 4).

All isolates were subsequently tested for *bla_{SPMI}* and *bla_{GIM}* gene and no amplicon of 400bp and 753 bp signifying *bla_{SPMI}* & *bla_{GIM}* respectively were detected. In this study combined disc test was considered as screening test for detection of metallo- β -lactamase activity in imipenem non-susceptible multi drug resistant isolates and PCR was considered as Gold standard test for MBL gene identification. Statistical analysis of these two qualitative tests was done by Chi square test (Table 5) using "Graph pad" statistical software.

P value was 0.020 and statistically significant correlation was there between two tests. Sensitivity was 88.89% (95% confidence interval 51.75% to 99.72%) and specificity 51.69% (95% confidence interval 40.84% to 62.41%). Positive Predictive Value was 15.69% (95% confidence interval 7.02% to 28.59%), Negative Predictive Value 97.87% (95% confidence interval 88.71% to 99.95%).

IV. Discussions

Wide spread usage of antibiotics exerts a selective pressure that acts as a driving force in development of antibiotic resistance. Thus the development of multidrug resistance in health care set up is very much common phenomenon. Resistance factors, particularly those carried on mobile genetic elements, can rapidly spread from patients to patients and are present both locally and globally. *Pseudomonas aeruginosa* harbouring MBL encoding gene emerge as carbapenem resistant as well as resistant to many other antibiotics of different classes. *bla_{vim}* and *bla_{IMP}* gene have been reported from various parts of the world [6].

Imipenem resistance is detected phenotypically in our institutions during routine laboratory work but genetic study report was lacking. So over a period of nine months from January 2012 to September 2012 our study searched for *bla_{vim}* and *bla_{IMP}* gene in Imipenem non-susceptible multidrug resistant isolates of *Pseudomonas aeruginosa*. We observed resistance to 2,3,4 and 5 another, different classes of antibiotics in Imipenem non susceptible isolates as was observed by Fereshteh Shahcheraghilet al [5].

For phenotypic identification of metallo- β lactamase activity combined disc test with imipenem (10 μ g) alone and imipenem-EDTA (10 μ g+750 μ g) combined disc were chosen as described by J.J.Pitout [8] and Yong et al 2000 [9]. The amount of EDTA as 750 μ g was selected from the studies experimenting with different amount of EDTA [9,10,11,12,13]. Ting-ting Qu et al (2009) [10] and L.Berges et al [11] showed that 292 μ g EDTA per disc gave only 3-4 mm increase in zone of inhibition during comparative study whereas 750 μ g and 930 μ g EDTA increases the zone-inhibition by more than 7mm. EDTA itself has an antibacterial activity. So higher dose of 930 μ g was not chosen [18]. The phenotypic study with 98 non-duplicate consecutive samples showed two groups--- MBL positive & MBL negative. 52.04% (51 out of 98) isolates appeared as MBL positive phenotypically and this data conforms with findings of other studies in our region [13,14,15,16].

Duplex PCR for *bla_{vim}* and *bla_{IMP}* was chosen as these two genes have been identified from all over the world. The present study showed existence of *bla_{vim}* gene only. PCR with designed primer detected existence of *bla_{vim}* gene in only eight 'Test samples' and in one 'Control' samples. *bla_{IMP}* gene was not identified in any of the strains. All isolates were further tested to confirm the absence of *bla_{SPMI}* and *bla_{GIM}* gene, which are less frequently seen beyond Brazil and Germany respectively [10, 12].

Comparing screening test (combined disc test) with gold standard PCR method, the sensitivity of MBL screening test appears 88.9% (95% CI 51.75%-99.72%), specificity 51.7% (95% CI 40.84% - 62.41%). The sensitivity of previous studies had varied from 89-100% [9,10,11,12,15] and in present study almost similar to them. The specificity of screening (combined disc) test is much lower than that obtained in other studies where it varied from 90- 100% [9,10,11,12,15]. The false positive screening test may have been resulted from synergistic effect of EDTA which acts not only as metal chelator but also increases membrane permeability and bactericidal effect [14]. Report showed high false positive results in case of Imipenem-EDTA disc as well as MBL E-test using EDTA with imipenem [12]. Yong D et al (2002) [11] commented in their study that with Imipenem and 750 μ g of EDTA the increase in zone of inhibition is generally 8-15 mm but in present study the increase in zone diameter is more than 20 mm in majority of the isolates. Use of another screening

test like MBL Etest or similar combined disc test using another carbapenem like meropenem might have reduced the rate of false positive result.

P-value of the 2×2 Chi-square test is 0.0202 which identifies statistically significant correlation between screening and gold standard test. Positive predictive value of the test is 15.69% (95% CI is 7.02% to 28.59 %) and Negative predictive value is 97.87% (CI 88.71% to 99.95). *bla_{vim}* gene in nine samples out of total 98 strains comprises of 9.18% only whereas study by Mariana Castenheira [17] with isolates collected from ten hospitals in and around Mumbai, India showed a value of 53.2% of *bla_{vim}* gene positive strains out of total 107 MBL producing *Pseudomonas* spp. The observation of that study confirmed that *bla_{vim}* gene was prevalent in India. In present study also *bla_{vim}* is the only gene detected. This finding also complies with the comment of Johann. D. D.Pitout [8] that usually a single MBL encoding gene is prevalent in a hospital or in particular geographical area. All of these isolates were obtained from pus samples collected from surgical site wounds and/ or primary wound in patients. This finding should be taken into account by clinicians in selection of antibiotics because carbapenem is one of the few antibiotics left for treatment of multidrug resistant *Pseudomonas aeruginosa* till date.

V. Conclusion

The tertiary care providing hospital deals with different types of patients in large number and in close contact. So the nosocomial infection with drug resistant strains of organisms is very frequent and often takes the form of outbreak due to treatment failure. Rapid identification of these microbes is essential. The study confirmed that *bla_{vim}* gene is not uncommon in eastern region of India. In consideration with global spread of these type of enzyme encoding genes the name of our country, India, may be included in the list of eastern Asian countries where these are prevalent. Unless we formulate a proper antibiotic policy and adhere to it strictly the risk of treatment failure will take rising graph endangering patients' lives.

VI. Conflict of interest

The author declares that there is no conflict of interest regarding the publication of this study.

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Table 1: Details of Primers used, annealing temperature and amplicon size

Primer name	Primer sequence (5' - 3')	Annealing temperature (Ta) ⁰ C	Amplicon size (bp)
IMP A IMP B	GAA GGC GTT TAT GTT CAT AC GTA AGT TTC AAG AGT GAT GC	40	587
VIM 2004A VIM 2004B	GTT TGG TCG CAT ATC GCA AC ATT GCG CAG CAC CAG GAT AG	54	382
SPM 1A SPM 1B	CTG CTT GGA TTC ATG GGC GC CCT TTT CCG CGA CCT TGA TC	40	400
GIM F GIM R	AGA AGC TTG ACC GAA CGC AG ACT CAT GAC TCC TCA CGA GG	40	753

Table 2 : Multi drug resistance to different classes of antibiotics in 98 imipenem non susceptible isolates of *P. aeruginosa*

	Resistance to 3 different classes	Resistance to 4 different classes	Resistance to 5 different classes	Resistance to 6 different classes
No of isolates	11	34	23	30
% of isolates	11.22	34.69	23.46	30.61

Table-3 Result of combined disc test with imipenem-EDTA and duplex PCR for *bla_{vim}* & *bla_{imp}* gene

	<i>bla_{vim}</i> present	<i>bla_{vim}</i> absent	<i>bla_{imp}</i> present	<i>bla_{imp}</i> absent
CDT positive(n)	8	43	0	51
CDT negative(n)	1	46	0	47
Total isolates	9	89	0	98

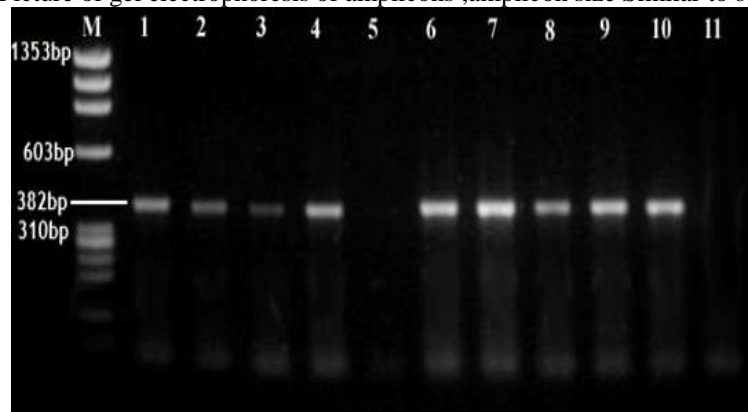
Table: 4 *bla_{vim}* gene in Imepenem non-susceptible multidrug resistant isolates of *P.aeruginosa*

Registration no.	specimen	Imepenem non susceptibility	Resistance to different (6) classes of antibiotic	CDT result	<i>bla_{vim}</i> gene
P32	pus	present	6	positive	present
P40	pus	present	5	positive	present
P61	pus	present	6	negative	present
P65	pus	present	6	positive	present
P76	pus	present	4	positive	present
P84	pus	present	6	positive	present
P89	pus	present	6	positive	present
P90	pus	present	5	positive	present
P96	pus	present	3	positive	present

Table -5 Comparative study between screening test (combined disc test) & Gold standard test (duplex PCR)

	Combined disc test Positive (n)	Combined disc test Negative (n)	Total isolates
<i>bla_{vim}</i> present (n)	8	1	9
<i>bla_{vim}</i> absent (n)	43	46	89
Total isolates	51	47	98

Figure1: Picture of gel electrophoresis of amplicons ;amplicon size Similar to *bla_{VIM}* gene



Representative PCR of eleven test samples harbored *bla_{VIM}* gene. M= ϕ X 174 DNA/ BsuRI (HaeIII) Marker (Fermentus);

Lane 1= P32 :Lane 2 = P40:Lane 3 = P61: Lane4 = P64: Lane5 = ATCC *P.aeruginosa* :Lane 6 = P76 :Lane7 = P84 : Lane8= P89:Lane9 = P90 :Lane10 P = 97 :Lane 11 = P51(negative sample)